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SKIN REACTIVITY AND SEROLOGICAL RESPONSE TO COCCIDIOIDIN SKIN TESTS*

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The coccidioidin skin test is an important diagnostic aid to the physician. Conversion from skin test negative to positive is pathognomonic and occurs early as a response to infection, often before serological tests become positive. Skin tests are sometimes repeated at weekly or bi-weekly intervals to determine whether conversion has occurred. Especially in areas where infection with *Coccidioides immitis* is endemic, skin tests with coccidioidin repeated at longer intervals are given for the purpose of epidemiological survey.

It has been reported that histoplasmin skin tests will induce a positive skin reaction (1). It has also been reported that a change from negative to positive serology, as well as enhancement of complement fixation titer, can occur as a result of a single diagnostic skin test with histoplasmin (2, 3). Since there is cross-reactivity between histoplasmin and coccidioidin with respect to their properties both as skin test and complement fixation antigens, it is important to evaluate whether skin testing with coccidioidin has a similar effect.

Smith *et al.* (4) performed a rigorous series of skin tests with 0.1 ml undiluted coccidioidin in human volunteers with no induction of positive skin sensitivity. Also, repeated skin tests did not stimulate production of complement fixing or precipitin antibodies. Rapaport *et al.* (5) in a series of weekly skin tests in 53 negative individuals reported 2 conversions to positive; one after 2 injections of 1:10 dilution and one following 3 injections of undiluted coccidioidin. Their data indicate anamnestic skin test responses to intradermal injection of 1:10 coccidioidin. In studies on the passive transfer of skin sensitivity to coccidioidin in man, Rapaport *et al.* (6) noted an enhanced efficacy of leukocyte extracts when injected intradermally in an equal volume of undiluted coccidioidin. Their data suggest the possibility of producing skin sensitivity in man by repeated injection of undiluted coccidioidin.

Recent studies on induction of skin sensitivity in guinea pigs and rabbits by subcutaneous injection of undiluted coccidioidins have indicated positive serological response demonstrable by agar gel immunodiffusion technics (7). This study therefore was designed to investigate the effect of repeated diagnostic skin tests on induction of skin reactivity and positive serology in both skin test negative and skin test positive individuals. In addition, the effect of same site injections on the size of reaction in skin test positive individuals was investigated.

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MATERIALS AND METHODS

Skin Tests

Twenty three volunteers previously determined to be positive or negative to the standard skin test dose of coccidioidin (0.1 ml of 1:100) participated in the study. Twelve skin test negative and eleven skin test positive individuals were injected with coccidioidin intradermally in the same site every other week until each individual had been skin tested three times. In each group, half of the subjects received Smith's coccidioidin Lot 64D4, a skin test antigen standardized in Dr. Smith's laboratory; the other half received Huppert's Lot XVB52F coccidioidin prepared for use as a complement fixation antigen and demonstrated to have skin test activity (8). The respective coccidioidins were injected intradermally in 0.1 ml aliquots at a point on the volar surface of the forearm 5 inches from the juncture of the wrist joint and the flexor digitorum. For the first two skin tests, both negatives and positives were injected with either Smith's coccidioidin diluted 1:100 or Huppert's coccidioidin diluted 1:40. In the third test, negatives received either Smith's coccidioidin in a 1:10 dilution or Huppert's diluted 1:4. Positive reactors were given two skin tests with coccidioidin of the same strength previously used; one in the same site and one on the other arm. All skin reactions were read at 24 and 48 hours. Both erythema and induration were measured and recorded in millimeters.

All participants in the study were bled by venipuncture at the study's initiation just before each skin test, and 5 weeks after the third skin

test. In addition, the positive individuals only were bled 5 days after their first skin test. Sera were drawn off within 24 hours, distributed to duplicate sterile bottles and stored at −20°C until the study was completed. This yielded a total of 103 serum specimens.

Serological Tests

Sera were tested by several agar gel double diffusion technics using three different coccidioidins. Macroimmunodiffusion plates were prepared according to the method of Huppert and Bailey (9). A micro-Ouchterlony technic was also employed utilizing commercially available Immunoplates[1] with a 7 mm center-to-center distance between the central and 5 peripheral wells.

An agar gel double diffusion tube test developed in our laboratories and currently being evaluated as a diagnostic aid in primary coccidioidomycosis can be briefly described as follows: 60 x 5 mm tubes were coated on the inside with 0.1% agar and dried at 37° C before use. A 10% coccidioidal antigen in 0.75% agar was layered in 0.2 ml amounts in the bottom of the tubes. An equal amount of plain agar was layered over the antigen in agar and upon gelation, 0.2 ml of the test serum was added, the tubes capped with modelling clay to prevent evaporation and incubated at 25° C for development of precipitin bands. Readings were taken daily and recorded at 72 hours. Proper lighting conditions for reading of plates, slides and tubes were maintained by the use of a specially constructed immunodiffusion viewing chamber with a circular fluorescent fixture on an adjustable platform. Sera were tested by macro plate, micro slide and tube technics utilizing two coccidioidins (XVB57FC, XVB60L) supplied by Dr. Milton Huppert of San Fernando Veterans Administration Hospital. Lot XVB57FC and XVB60L are culture filtrate and lysate coccidioidal antigens respectively and are prepared according to the method of Huppert and Bailey (10).

The 103 serum specimens were also evaluated for precipitin blocking antibodies by the agar gel precipitin inhibition (AGPI) method of Ray (11). In this method a culture filtrate coccidioidal antigen is titrated by Ouchterlony technic against hyperimmune monkey serum to obtain a minimum reacting dilution of each. Serial twofold dilutions of the sera to be tested are then incubated at 37° C for one half hour in the minimum reacting dilution of antigen. Subsequent testing of the incubated antigen-serum dilutions against the minimum reacting dilution of the monkey reference serum produces an inhibition titer. Preliminary results on clinical coccidiomycosis serum (AGPI) titers indicate close correlation with complement fixation titers (12).

[1] Hyland Laboratories #85-072, Pattern B.

RESULTS

Skin Tests

Data on 5 positive individuals injected with Smith's Lot 64D4 skin test antigen indicates a reduced reaction area on third retest when the antigen is administered in precisely the same site three times at two-week intervals. A virgin site tested simultaneously with the third re-test site showed a larger induration at both 24 and 48 hours. Figure 1 indicates the results obtained from 5 individuals injected with Smith's Lot 64D4 coccidioidin and 6 injected with Huppert's Lot XVB52F coccidioidal antigen. Results with Huppert's antigen were strikingly different, the virgin site showing less induration than the retest site at both 24 and 48 hours.

Although those selected to receive Huppert's antigen all had previous positive skin reactions to standard strength coccidioidin (1:100), only one individual (3+ on previous testing) reacted with induration of 15 × 18 mm at 48 hours. Since the 1:40 dilution had been stored at 5° C for over 6 months, a freshly prepared 1:40 dilution was used for the second and the two third skin tests in all six individuals. Table I indicates areas of induration in mm for the eleven positive individuals.

Skin Test Negative Subjects

Of 12 individuals negative to 1:100 and 1:40 dilutions of the respective coccidioidins given twice in the same site at two week intervals, there were 3 who reacted positively to a same site injection of a 10 × dose of the respective coccidioidin. Approximately 10 months later all 3 individuals were re-tested with standard strength coccidioidin (Smith's Lot 64D4 1:100) and were all negative. One week later 2 of the 3 were again tested with 1:10 and skin reactivity measured at 24 and 48 hours. Induration responses to the 1:10 (10×) coccidioidin are given in Table II.

Serological Tests

Ouchterlony tests using both a "lysate" and a culture filtrate coccidioidal immunodiffusion antigen (10) were performed by macro petri plate and micro slide technics. Results

with each antigen were uniformly negative on the 55 serum specimens from positive skin reactors as well as the 48 sera from the negatives.

Results of the agar gel double diffusion tube test using both the culture filtrate and lysate antigens are given in Table III. Tubes were read at 72 hours by three different people

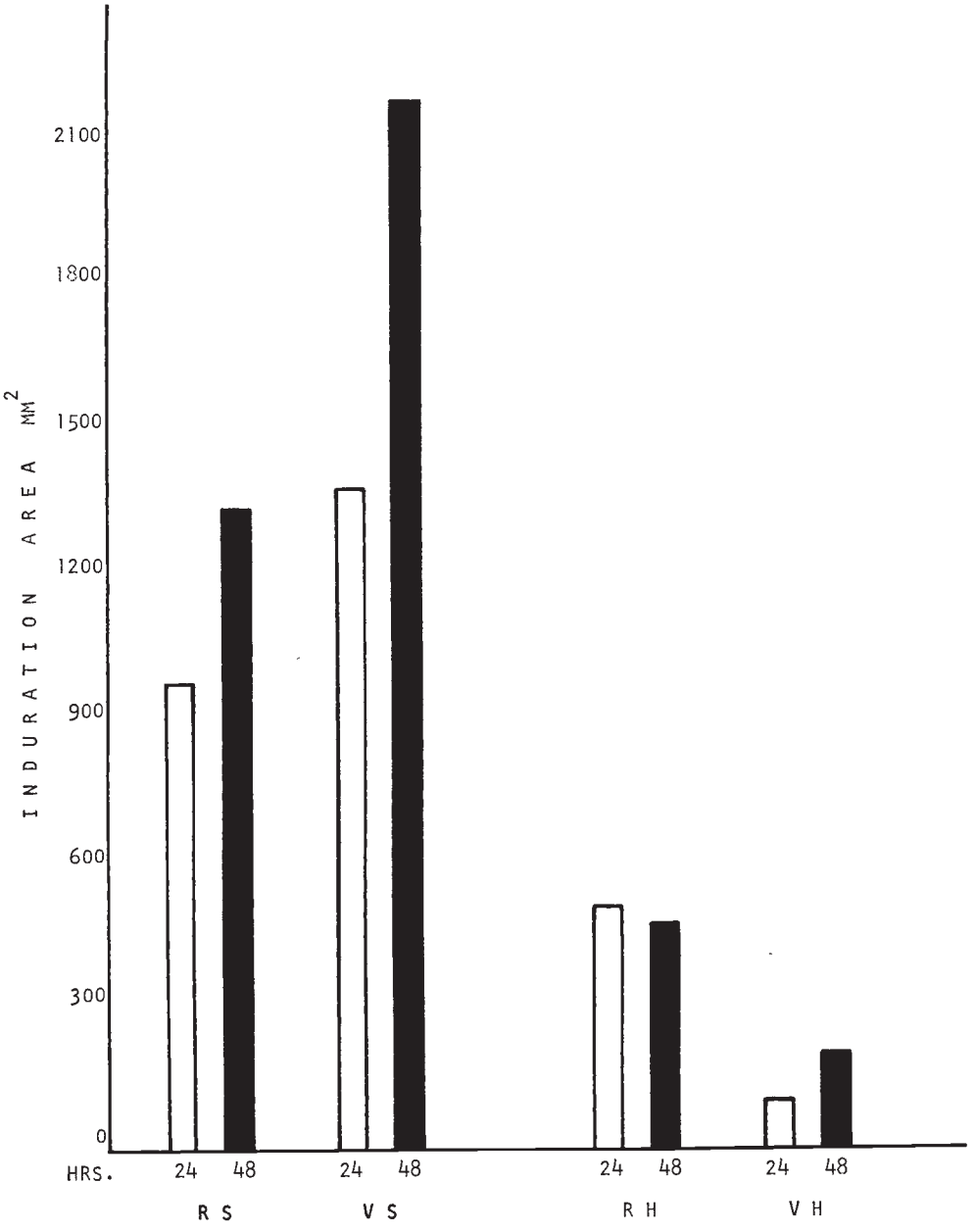

FIG. 1. Comparison of retest and virgin site induration areas in coccidioidin skin test positive individuals.

R = retest site
S = Smith's Lot 64D4 1:100
V = Virgin site
H = Huppert's XVB52F 1:40

TABLE I

Areas of induration† in mm in response to intradermal injection of 0.1 ml of either Smith's Lot 64D4 coccidioidin or Huppert's Lot XVB52F Coccidioidal antigen

Coccidioidin	Retest Site						Virgin site	
	I		II		III		III	
	24 Hr	48 Hr	24 Hr	48 Hr	24 Hr	48 Hr	24 Hr	48 Hr
Smith's 64D4 1:100								
R V L	50 × 35	40 × 36	41 × 50	50 × 32	40 × 30	26 × 35	52 × 46	40 × 38
E B W	30 × 21	40 × 30	30 × 36	36 × 50	19 × 18	0 × 0	38 × 40	45 × 45
W M C	25 × 23	33 × 30	47 × 47	50 × 62	35 × 30	48 × 69	32 × 24	49 × 52
H A R	20 × 20	36 × 40	30 × 28	30 × 31	29 × 29	24 × 21	12 × 12	29 × 44
I B S	20 × 20	40 × 40	10 × 11*	20 × 25*	35 × 42	47 × 43	43 × 49	60 × 60
Huppert's XVB52F 1:40								
G D W	0 × 0	0 × 0	23 × 23	20 × 30	28 × 20	20 × 23	0 × 0	19 × 17
V E N	0 × 0	0 × 0	5 × 5	15 × 15	15 × 15	0 × 0	0 × 0	8 × 8
D D W	0 × 0	0 × 0	20 × 17	17 × 17	9 × 9	9 × 9	0 × 0	0 × 0
D R K	0 × 0	0 × 0	11 × 12	5 × 5	6 × 5	0 × 0	0 × 0	5 × 5
D E T	10 × 10	15 × 18	0 × 0	13 × 16	29 × 30	41 × 44	24 × 25	25 × 30
C H W	0 × 0	0 × 0	18 × 17	23 × 21	34 × 37	22 × 22	0 × 0	0 × 0

* 0.05 ml only injected.

† In previously determined skin test positive reactors.

TABLE II

Induration Response in mm to 10× Coccidioidin in skin reactors negative twice before in the same site to 1× Coccidioidin

	3rd Injection			10 Months Later New Site	
	Coccidioidin Dilution	24 Hr.	48 Hr.	Coccidioidin Dilution	24 Hr.	48 Hr.
R O B	Smith's 64D4 1:10	17 × 17	15 × 12	Smith's 64D4 1:10	Not done	Not done
C A H	Smith's 64D4 1:10	28 × 25	20 × 20	Smith's 64D4 1:10	6 × 6	5 × 5
R O T	Huppert XVB52F 1:4	17 × 13	10 × 9	Smith's 64D4 1:10	15 × 12	3 × 3

TABLE III

Numbers of ± results (numerator) in total (denominator) serial serum specimens tested by the agar gel double diffusion tube technique using two immunodiffusion coccidioidal antigens

Skin reactors	Culture filtrate antigen				
Time of bleeding post skin test*	0	I + 5 days	I + 2 wks	II + 2 wks	III + 5 wks
Positives	1/11	2/11	2/11	0/11	0/11
Negatives	0/12	N.D.	0/12	0/12	0/12
	Lysate Antigen				
Positives	6/11	7/11	8/11	7/11	8/11
Negatives	2/12	N.D.	1/12	1/12	3/12

* 0, I, II, III refer to preinjection and 1st to 3rd skin test injections respectively.

and ± results were recorded as such only if 2 of the 3 agreed. Precipitin bands in these tubes were usually closer to the serum meniscus and were more diffuse than occurred with reference positive human or rabbit serum. They were also difficult to distinguish from non specific bands.

The agar gel precipitin inhibition (AGPI) test using hyperimmune monkey serum as a reference and a coccidioidin prepared by John Converse of Ft. Detrick, Md. was negative for the 48 and 55 serial serum specimens from negative and positive skin reactors respectively[2].

[2] We are indebted to Dr. John Ray, Jr. for testing the 103 serum specimens in his laboratory. The coccidioidin was prepared from culturing the Cash strain of *Coccidioides immitis* in a liquid medium with ammonium lactate as a nitrogen source. (13). Subsequent to receipt of this manuscript for publication, Dr. Ray has indicated some positive results have been obtained using a different coccidioidal antigen and method.

DISCUSSION

Same Site Reactions in Positive Reactors

Although there is considerable individual variation in re-test site induration among the 5 subjects injected with 1:100 Smith's Lot 64D4 coccidioidin, all responded with definite positive reactions if the recommended pro-

cedure of reading at both 24 hours and 48 hours was followed (14). Under the experimental conditions described desensitization of the test site did not occur to any appreciable extent. The variable results with Huppert's coccidioidal antigen, however, call into question its efficacy as a skin test antigen. Loss of skin test activity, on storage of diluted coccidioidin, has been reported (4). This suggests a reasonable explanation for lack of response in 5 of 6 individuals to the first injection. A freshly prepared 1:40 dilution was used for the second and the two third injections. Although response in the re-test site was adequate in 5 of the 6, if 24 hour and 48 hour readings were made, only 3 of the 6 showed a positive response at the virgin site. Since re-test site and virgin site injections were made with the same preparation, two previous injections may have conditioned the site in some way toward an enhanced response.

Same Site Reactions in Negative Reactors

Although 3 of the 12 skin test negative individuals reacted positively when tested with Smith's 1:10 or Huppert's 1:4 coccidioidin following two previous same site negative tests with 1:100 or 1:40 of the respective antigens, the positive skin reactions were most probably due to low grade skin hypersensitivity which was enhanced by the testing procedure. Rapaport *et al.*, (5) have reported enhancement of skin reactivity with skin tests repeated at weekly intervals.

In this highly endemic area, it is perhaps possible but highly improbable that the subjects, during the course of the experimental procedure, were exposed to *C. immitis* and acquired a subclinical infection. Under such circumstances, however, reactions to standard strength 1:100 coccidioidin are usually definite and skin sensitivity is of long duration (14). All 3 subjects tested ten months later were negative to 1:100 coccidioidin, but 2 re-tested with 1:10 coccidioidin retained their reactivity to a 10× dose.

Serological Results

The predominantly negative serological results confirm findings reported by Smith *et al.*, (4) and Sigrest *et al.*, (2) among others. Repeated skin testing *per se* with standard strength coccidioidin does not produce a humoral antibody response as is the case with histoplasmin. More sensitive technics such as passive cutaneous anaphylaxis (PCA) or passive hemagglutination might be successful in demonstrating an antibody response but are of limited practical use in clinical coccidioidomycosis serology.

Results with the agar gel tube technic, especially with the lysate coccidioidal antigen, suggest further evaluation of this technic is warranted.

SUMMARY

The contribution of diagnostic coccidioidin skin testing to altered skin reactivity and positive serology was investigated in positive and negative skin reactors using Smith's Lot 64D4 skin test coccidioidin and Huppert's XVB52F coccidioidal antigen.

Data on skin test positive individuals indicates a reduced reaction area when the antigen is administered in precisely the same site three times at two-week intervals. Results with Huppert's coccidioidal antigen were variable and can be attributed to a loss of potency upon storage.

Three of twelve skin test-negative individuals had positive skin reactions when tested with Smith's 1:10 or Huppert's 1:4 coccidioidin following two same site negative tests with 1:100 or 1:40 of the respective antigens. These positive skin reactions have been interpreted as being due to preexisting low grade skin hypersensitivity which was enhanced by the testing procedure. Attempts to demonstrate humoral antibody in serial serum specimens by several sensitive immunodiffusion technics were unsuccessful. Repeated skin testing *per se* with standard strength coccidioidin does not produce a humoral antibody response.

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